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ABSTRACT| Carbon nanotubes have several properties that make them optimal for use in biomimetic systems. The carboxyl groups around the opening of the tube allow for functionalization of the nanotube. Their size is compatible for use in biological membranes. However, before carbon nanotubes can be usefully incorporated into lipid membranes, two things need to be known: their exact size and the number of nanotubes that will be reconstituted. To determine the size, the nanotubes were put into solution with detergent micelles (Triton x-100) in order to break up the aggregated bundles before being exchanged using dialysis membranes with another detergent (DTAB) which has a smaller micelle size. In order to quantify the number of carbon nanotubes in the lipid membranes, the carboxyl groups were functionalized with Au-Fab-FITC which can be quantified using UV-Vis spectroscopy. From there a lipid assay was performed to measure the amount of lipid in the samples, suggesting 71.3 tubes per vesicle.

INTRODUCTION

Carbon nanotubes (CNTs) are long, hollow tubes with an opening of about 1.5 nm. The length can vary from a few nanometers to a few microns depending on the preparation. The side walls of CNTs are highly hydrophobic and the openings of the tubes have carboxyl groups that allow for the functionalization of the nanotubes.

Currently work is being done to explore methods to cut CNTs into smaller sizes that are similar to proteins. These shorter CNTs can be incorporated into lipid membranes to act as biological molecules. In order for this to be a useful process however, two things need to be known. A method must be developed to determine the size of these shorter nanotubes. Also, a way to quantify the number of CNTs that are incorporated into the membrane is needed.

CARBON NANOTUBE SIZING

Before CNTs are incorporated into the lipid membranes, the length of the tubes must first be determined. In order to size sub-micron particles that are suspended in solution, a method

called dynamic light scattering (DLS) is used. DLS works by measuring the Brownian motion of a system through light scattering. Brownian motion is the random movement of suspended particles due to collisions with surrounding particles in the medium. Larger particles will have much slower Brownian motion. The two light scattering theories that can be used are Rayleigh scattering or Mie Theory. Rayleigh scattering is used when the particles being sized are much smaller than the wavelength of light being used. Mie Theory is more useful as the size of the particles approaches the wavelength of light. Then, the DLS will use the Stokes-Einstein equation $d(H) = \frac{kT}{3\pi\eta D}$ where k is Boltzmann's constant, T is the temperature, η is viscosity and D is the translational diffusion coefficient to find $d(H)$, the hydrodynamic diameter. It assumes the particles are spherical in shape. CNTs are in fact cylinders, so the diameter of the "sphere" measured is assumed to be the length of the carbon nanotube.[1]

There is a problem in trying to measure CNTs. The native complex will naturally aggregate, forming bundles of CNTs (Figure 1A). This makes it hard to accurately size the CNTs because there are no single tubes in solution.

In order to combat this problem, detergent was added to the CNT complex. Detergents are amphiphilic molecules meaning that they are both hydrophobic and hydrophilic. The tail end is hydrophobic just like the side walls of the nanotubes. When detergent micelles are introduced to the solution (Figure 1B), the monomers of detergent begin to work their way between the CNTs. As the concentration of detergent increases (Figure 1C), the bundles begin to break apart into individual CNTs and micelles begin to form around each tube. Once the concentration of detergent is above the critical micelle concentration (Figure 1D), the tubes will be completely separate and each nanotube will be wrapped in detergent micelles.

Materials and Methods

The detergents used in this study were sodium dodecyl sulfate (SDS) (GIBCO), Triton x-100 (TX100) (ThermoScientific), and dodecyltrimethylammonium bromide (DTAB) (Alfa Aesar). The concentrations used were SDS (3%), TX100 (1%), and DTAB (0.6%).

Carbon nanotubes in detergent solution

Detergent solutions were prepared at higher stock concentrations in water and were filtered through a syringe filter (0.2 μm). The detergents were diluted to the listed concentration in a 400 μL sample containing CNT complex (160 μL) and the necessary amounts of detergent and water. The solutions were mixed gently at room temperature overnight before being sonicated in a bath sonicator for 15 minutes and immediately measured on the DLS.

Carbon nanotubes with detergent solution exchange

TX100 solution was prepared as before and filtered through the 0.2 μm membrane. The detergent was diluted to 1% in a 400 μL sample containing CNT complex (160 μL) and the necessary amounts of TX100 and water. The solution was mixed gently at room temperature overnight. A DTAB solution (~200 mL) was prepared at 0.6% and filtered through a 0.2 μm

membrane. The TX100 was exchanged for DTAB using a 2000 kDa MWCO dialysis membrane (ThermoScientific). The sample was exchanged overnight at room temperature while gently stirring the outside DTAB solution. The sample was removed from the dialysis cassette and sonicated for 15 minutes before immediately being sized using the DLS.

Results

Carbon nanotubes in detergent solution

For the detergent SDS the average micelle size is 1.502 nm in diameter (Figure 2A). The average size of the CNT complex is 46.87 nm. When the CNT complex is put into solution with the SDS detergent solution, however, the size stays at 46.87 nm. This suggests that the detergent is not successfully entering the CNT bundles to break apart the nanotubes. DTAB (Figure 2B) shows a very similar trend. The micelle size is 3.548 nm but the mixture of CNTs in DTAB is 302.6 nm, showing that the bundles have not been broken up. In contrast, the size of the TX100 micelles is 8.648 nm (Figure 2C). Unlike the other two detergents, the size of the CNT complex in TX100 is 8.080 nm. This is much smaller than the complex size of 46.87 nm, suggesting that the bundles have in fact been broken up. A new problem arises however because the peak of the detergent micelles cannot be distinguished from the peak of the CNT complex wrapped in detergent micelles. The TX100 was the only detergent successful in breaking apart the aggregated bundles of nanotubes but the micellular size is too large to be useful in the sizing protocol.

Carbon nanotubes with detergent solution exchange

In order to combat this problem a detergent exchange was performed. The CNT bundles were broken up in the TX100 and then exchanged for DTAB which has a smaller micelle size. When the CNT complex was put into a DTAB solution with no detergent exchange, the size of the particles was measured at 302.6 nm (Figure 2D). Once the CNTs were broken up with TX100 and then wrapped in DTAB micelles the size of the molecules decreased quite a bit. The two peaks presents were 22.7 nm and 130.1 nm. Both of these are markedly smaller than the 302.6 nm peak from before showing that the micelles were broken up by the TX100 and remained broken up by the DTAB with its smaller micelle size. It should be noted that when watching the measurements of the DLS over time the solution was visibly reaggregating over time. The 22.7 peak was decreasing in height and the 130.1 peak increasing. For this reason, an immediate measurement on the DLS is necessary.

CARBON NANOTUBE QUANTIFICATION

Once these smaller CNTs are prepared and sized, they can be incorporated into lipid membranes as seen in Figure 3A. In order for this incorporation to be useful, the number of tubes that are incorporated into the lipids must be known.

As mentioned previously, there are several carboxyl groups present on the opening of each CNT which allow for the functionalization of the CNTs. Carboxyl groups will react with

cysteamine to form a thiol group (Figure 3B). Gold has a high affinity for thiol which allows for the conjugation of a gold-Fab-FITC fluorophore to the exposed thiol group (Figure 3C). As FITC has a UV-vis signature, the amount of the fluorophore can be quantified using UV-Vis spectroscopy and allowing for the quantification of the CNTs in each vesicle.[2]

Materials and Methods

CNT-LUV preparation

The lipids used in this experiment were 18:1 *Δ9-Cis* 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids) and Texas Red® 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (TxRed) (Life Technologies).

For the lipid only control, 99.5% DOPC and 0.5% TxRed was dried overnight *in vacuo* at 2 mg/mL. The sample was rehydrated with PBS buffer (1 mL). The sample was freeze thawed for 10 cycles and was extruded to 200 nm with an extruder membrane.

For the CNT-LUVs, 99.5% DOPC and 0.5% TxRed was dried overnight *in vacuo* at 3 mg/mL. The sample was rehydrated with PBS buffer (1 mL). The sample was freeze thawed for 10 cycles and was extruded to 200 nm with an extruder membrane. To incorporate the CNT complex, the LUVs just prepared (500 μL) was mixed with CNT complex (500 μL) and incubated overnight at 4°C. The sample was extruded to 200 nm with an extruder membrane.

CNT functionalization assay

To determine the amount of CNTs in a sample, the CNT-LUVs are reacted with cysteamine (41 μL, 1 μg/mL) (Sigma Aldrich) and incubated for 5 hours at room temperature. The free thiols were reacted with Au-Fab-FITC (55 μL, mg/mL) (Life Technologies) and stored overnight at 4°C. An SEC column was run in PBS buffer to separate all unbound reagents. The aborbance was measured at A_{495} to quantify the number of CNTs.

Lipid assay

To determine the amount of lipid in solution, the samples from the functionalization assay were evaporated of all solvents and then reconstituted in chloroform (2 mL) (Sigma Aldrich). Ammonium ferrothiocyanate (2 mL) was added and the solution was vortexed for 1 minute. The ammonium ferrothiocyanate was prepared by mixing ammonium thiocyanate (30.4 g) (Sigma Aldrich) and iron (III) chloride hexahydrate (27.03 g) (Sigma Aldrich). Then, the bottom reddish layer was removed and the absorbance was measured at A_{488} to quantify the lipid. A standard curve was prepared with known concentrations of lipid in chloroform.[3]

Results

Standard Curve

Figure 4A shows the absorbance at 488 nm for the standards prepared for the standard curve. Figure 4B fits this data to an exponential curve and gives the equation for this standard curve.

CNT functionalization assay

Figure 4C shows the absorbance spectra for the CNT functionalization assay. At 495 nm, the absorbance of the CNT-LUV fraction was much higher than the absorbance of the LUV only fraction. This higher absorbance is due to the binding of the Au-Fab-FITC to the CNTs in the membrane.

Lipid assay

In order for the number of CNTs in the sample to mean anything, first the amount of lipid in the sample must be known. Figure 4D shows the lipid assay quantifying the amount of lipid in the sample. The absorbance at 488 nm and therefore the lipid concentration of the CNT-LUV fraction and the LUV only fraction is the same. This means that in the CNT quantification assay, the higher signal at 495 nm is definitely due to the binding of the fluorophore and not due to a higher concentration of lipid.

Calculations of nanotubes per vesicle

From the functionalization assay, the absorbance of the LUV only fraction was subtracted from the absorbance of the CNT-LUV fraction. From there, Beer's Law and the fact that there are 2.9 FITC per Fab and 1 Au per Fab allow for the calculation that there are $3.73 * 10^{11}$ Au molecules in the sample.

From the lipid assay, the absorbance of the CNT-LUV samples was converted to the amount of lipid in the sample using the standard curve equation. From there, the number of vesicles can be determined. Dividing the number of gold molecules by the number of vesicles gives a final value of 71.3 CNTs per vesicle.

CONCLUSIONS

From these experiments it can be concluded that there is now a method to accurately size CNTs in solution, using detergent exchange to break up the bundles and wrap each tube in small micelles. It can also be concluded that there are approximately 71.3 CNTs incorporated into the lipid membrane of the vesicles.

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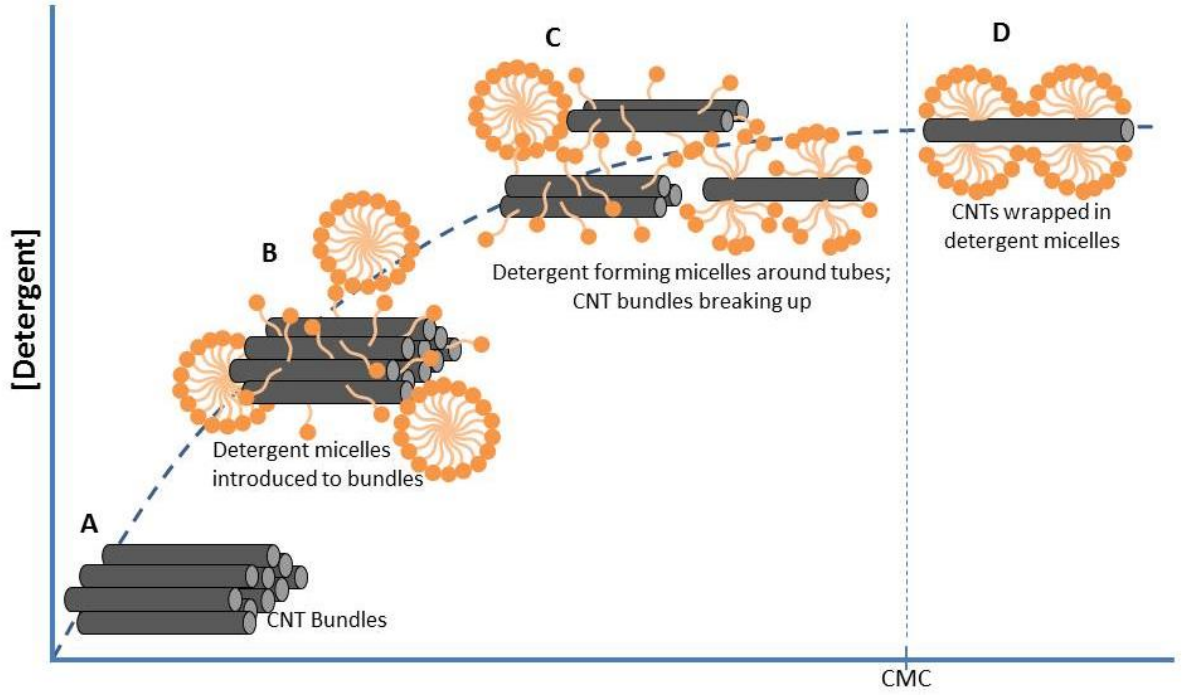

Figure 1| (A) CNT complex in its native, aggregated form (B) Detergent micelles begin to be introduced to the sample. The hydrophobic tails are attracted to and begin to stick to the hydrophobic side walls of the CNT. (C) The detergent monomers begin to break up the bundles and start to form micelles around the individual tubes. (D) CNTs are completely broken up and each tube is wrapped individually in micelles.

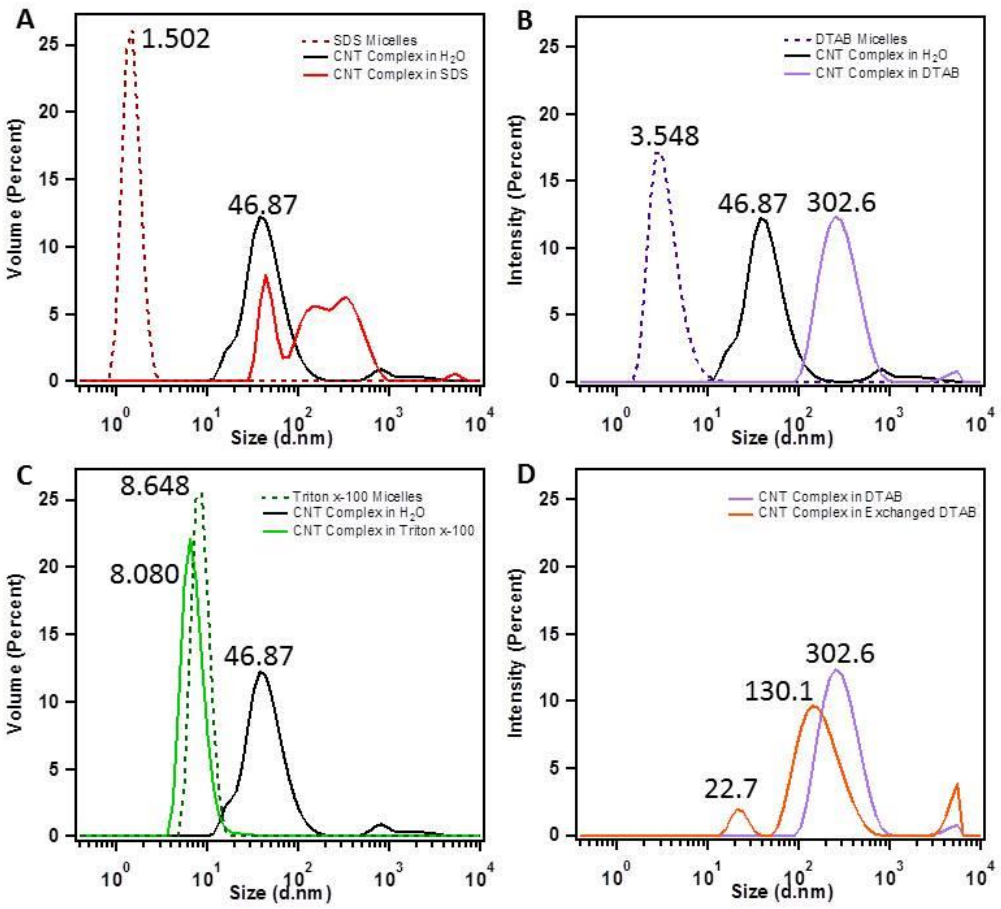

Figure 2| (A) SDS micelles have a diameter of about 1.502 nm, the CNT complex has a diameter of about 46.87 nm, and the CNT complex in SDS has a diameter of about 46.87 nm. The aggregated bundles are not broken up by the detergent. (B) DTAB micelles have a diameter of about 3.548 nm, CNT complex has a diameter of about 46.87 nm, and the CNT complex in DTAB has a diameter of about 302.6 nm. The aggregated bundles are not broken up by the detergent. (C) Triton x-100 micelles are about 8.648 nm in diameter, the CNT complex is about 46.87 nm in diameter and the CNT complex in TX100 is about 8.080 nm in diameter. The aggregated bundles are broken up by the detergent. (D) CNT complex in DTAB have a diameter of 302.6 nm. After the exchange of TX100 for DTAB, the diameter is measured at 22.7 nm and 130.1 nm. The aggregated bundles were broken by the TX100 and remained broken when wrapped with DTAB.

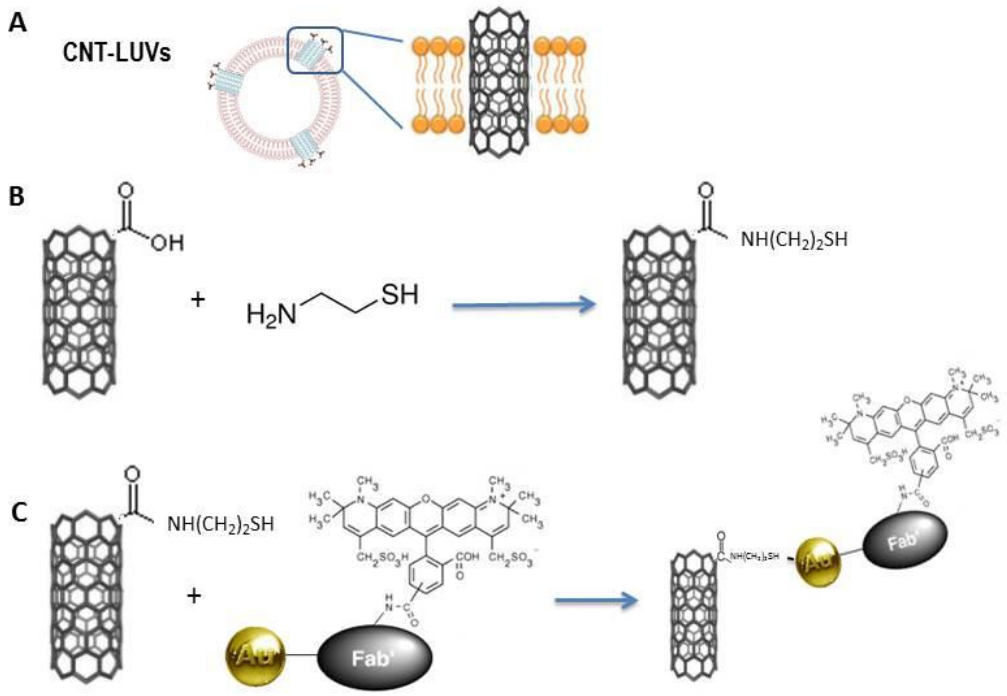

Figure 3| (A) CNT incorporation into the lipid membrane (B) Carboxyl group of CNT reacts with cysteamine to form a thiol. (C) Thiol group will conjugate with an Au-Fab-FITC fluorophore.

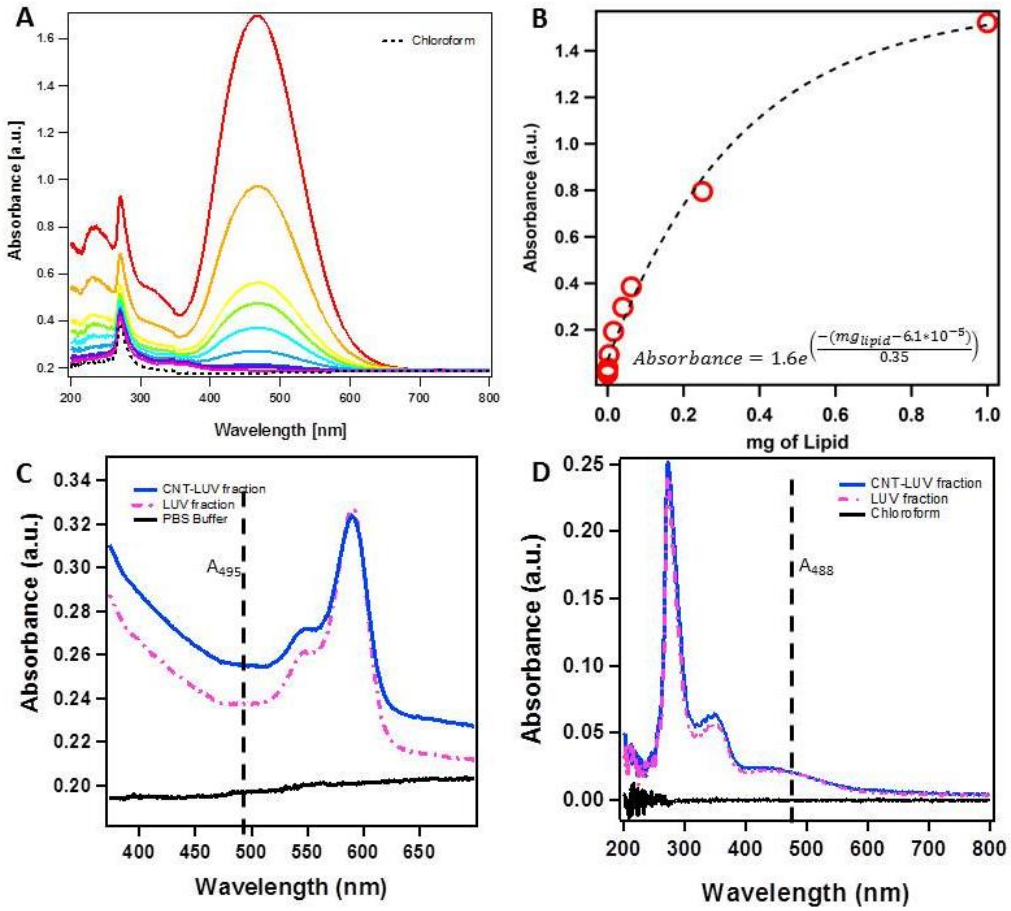

Figure 4| (A) UV-Vis absorbance spectra for the standards prepared for the lipid assay quantification (B) Standard curve calculated from the absorbance at 488 nm for the lipid assay (C) CNT functionalization assay showing the absorbance at 495 nm for both CNT-LUVs and LUVs. Au-Fab-FITC emission is at 495 nm. (D) Lipid quantification assay showing the absorbance at 488 nm for the CNT-LUVs and the LUVs.